Stressed Out!
Effects of Bisphenols on the Cellular Stress Response

A Major Qualifying Project
Submitted to the Faculty of
Worcester Polytechnic Institute
in partial fulfillment of the requirements for the
Degree of Bachelor of Science
in
Biology & Biotechnology
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Date: 26 April 2018
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Abstract

Concern over the safety of BPA in plastic products has lead to its substitution with related bisphenols BPF and BPS, though the safety of these alternatives is unknown. Using fluorescence microscopy and Western blots, we examined cellular stress responses in several human cell lines following treatment with bisphenols. We find that BPA and BPF cause stress granule formation, likely via activation of the PKR kinase pathway. This work has important implications for the continued use of bisphenols in plastics.

Acknowledgements

Our projects success depended on the generous contributions of many individuals and institutions. Our team would like to thank the following parties:

Professor Natalie Farny for her continued guidance and support throughout the duration of our project. We certainly could not have achieved our research goals without her expertise.

Professor Jill Rulfs for generously donating the T47D line, without which our project would not have been possible.

Dr. Nancy Kedersha and Dr. Marta Fay of Brigham and Women's Hospital for gifting us with the U2OS-DS cells and the HAP1 cell lines, without which our project would not have been possible.

Worcester Polytechnic Institute Department of Biology and Biotechnology for providing our team with space, supplies, and equipment in the Biology Project Lab.

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Background

Cellular Stress Response

As eukaryotic cells live and grow, they encounter many different environmental stresses to which they must adapt, or at least temporarily endure, in order to survive. Some common stressors include thermal stress, oxidative stress, viral infection, and heavy metal stress, among others (Kültz 2003). As a response mechanism to such a wide variety of potential stresses, eukaryotic cells have developed the cellular stress response (CSR). The CSR encompasses the cellular processes that occur when a cell's macromolecules are damaged to a significant level (Kültz 2003). During this response, various cellular pathways are triggered that collectively work to evaluate, prevent, and reverse damage to the cell, and temporarily increase the cell's ability to tolerate stress (Kültz 2003; Kültz 2005). If damage to the cell is too severe to repair, the CSR will induce the cell to undergo apoptosis (Kültz 2003). Different portions of the CSR act over different time frames. Short-term responses are fast and focus on managing damaged macromolecules (Kültz 2003). The long-term CSR is much slower and is focused on restoration of cellular homeostasis (Kültz 2003; Kültz 2005). During the initial stage of the CSR, cell growth is arrested, nucleic acids and salvageable proteins are repaired, and any macromolecular debris is cleared (Kültz 2003). This stage of the response is stress-specific and involves the formation of stress granules and processing bodies (Kültz 2005).

Stress Granules

Stress granules (SGs) are transient structures that form inside cells during a cellular stress response that may be caused by a wide variety of stressors including infection, toxicity, and nutrient deficiency (Donnelly *et al.* 2013). They contain the mRNAs from stress-induced translational arrest as well as certain proteins associated with stress response, some of which are common to all SGs such as G3BP and TIA-1, and some which depend on the mechanism of stress induction. The mRNAs stalled from translation are released from the polysomes, and are packed into cytoplasmic foci to form the SGs (Aulas *et al.* 2017). The translation-related proteins from the disassembled polysomes are sorted, and some are also packed into the SGs (Kedersha *et al.* 2008). These polysomes, i.e. messenger ribonucleoprotein particles (mRNPs) are reorganized before packed into SGs (Kedersha *et al.* 2013).

One common SG formation mechanism is via phosphorylation of eukaryotic translation initiation factor 2α (eIF2α). Under normal conditions, eIF2α is responsible for the delivery of initiator tRNAs to pre-initiation complexes required for mRNA translation. During a stress response, certain kinases are activated and phosphorylate eIF2α at serine 51 (S51) position, thus preventing translation initiation (Aulas *et al.* 2017). This results in a number of untranslated mRNAs accumulating in the cell, which then become components of the stress granules. There are four known kinases that can perform this phosphorylation event in mammals, including protein kinase R (PKR), general control non-repressed 2 kinase (GCN2), heme-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK) (Basu *et al.* 2017). These kinases have

been associated with responses to viral infection, nutrient deprivation, heme deficiency, and endoplasmic reticulum stress, respectively (Basu *et al.* 2017).

Processing Bodies

   Processing bodies (P bodies, or PBs) are also self-assembled cellular structures formed in response to stress. They are related to the SGs and share some similarities, but also have several distinct characteristics. PBs contain several factors involved in mRNA decay, while SGs recruit translation initiation factors (Kedersha *et al.* 2008). PBs and SGs can interact with each other when, for example, certain mRNA transcripts in SGs may be transported to PBs for degradation (Kedersha *et al.* 2008).

Bisphenol A

   Cell responses can be activated by a number of stressors; heat shock, viral infection, oxidative stress, and toxins all initiate a cell stress response. Bisphenol A (2,2-bis (4-hydroxyphenyl) propane, commonly known as BPA) is a pervasive chemical compound that causes damage to DNA (Meeker *et al.* 2010). BPA is a synthetic organic compound composed of a pair of connected phenol rings. Initially discovered in 1890, it was not widely used in plastic manufacture until the 1960's. It is a major component in the manufacture of epoxy resins and polycarbonate plastics, as well as less common polyacrylates and polyesters. Epoxy resins are used widely in automotive parts, PVC piping, can coatings, adhesives, and dental sealants while polycarbonate plastics are found in household appliances, food packaging, bottles, plastic wraps, packages, and utensils (Kang *et al.* 2006).

   Currently over 6 billion pounds of BPA are produced per year worldwide, with an additional 100 tons released into the atmosphere as a byproduct of the production process. BPA has one of the highest volumes of production of any chemical manufactured today (Vandenberg *et al.* 2007). It can be detected in most available environmental sources, including groundwater, river water, the atmosphere, and soil (Kang *et al.* 2006). Despite its environmental prevalence, most BPA intake is still a result of plastic leaching from food packaging into the food it contains. BPA that enters the human system is generally metabolized to BPA glucuronide (BPA-g) by the liver, then removed in urine (Lacroix *et al.* 2011). The BPA that is not metabolized and excreted can be detected in human serum, amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood. BPA is so prevalent that it has been shown to be detected in upwards of 90% of urine samples in a variety of sample populations from different ages, genders, and geographic regions (Vandenberg *et al.* 2007).

   BPA that is not excreted from the human system functions as a toxin in many pathways, and has been linked to a number of chronic diseases including diabetes, obesity, cardiovascular disease, chronic kidney disease, birth defects, developmental disorders, respiratory diseases, behavior disorders, autoimmune diseases, and breast cancer. BPA is linked to many diseases, but operates in them all through five mechanisms; endocrine disruption, genetic damage, epigenetic effects, oxidative stress, and cell signaling (Rezg *et al.* 2014).

Endocrine disruptors chemically interfere with the natural hormone signaling system by altering the secretion of hormones, interfering with hormone-receptor interaction, or modifying the metabolism of circulating hormones (Rezg *et al*. 2014). BPA was first identified as an endocrine disruptor in the 1930's during a search for a synthetic estrogen alternative. Inside the cell, BPA mimics the function of estrogen, binding to estrogen receptors alpha and beta (with a particular affinity for ERβ) and interfering with the normal hormone-receptor interaction (Routledge *et al*. 2000). Estrogen receptors regulate cell proliferation and cell stress response pathways - their overstimulation can lead to diverse effects in the organs that estrogen targets such as the brain, ovaries, mammary glands, and uterus (Vandenberg *et al*. 2009).

Genetic damages can be caused by DNA damage or chromosomal aberrations (aneuploidy, chromosomal segmentation). In vitro and in vivo studies have shown BPA to induce DNA adducts, aneuploidy, and mutagenicity, which contribute to infertility (in both sexes), miscarriages, and birth defects (Rezg *et al*. 2014). Additionally, genetic damage activates the cell stress response.

Epigenetic effects on the cell involve mechanisms of gene regulation that produce changes in gene expression without changing the genetic code. This is achieved through DNA methylation, histone modification, and expression of ncRNAs. BPA alters pathways by two of these mechanisms, mainly methylation and ncRNAs. Specifically, it has been shown to cause hypermethylation of estrogen receptors, hypomethylation of CpG (cytosine-guanine dinucleotide) islands in DNA, hypermethylation of CpG islands in genes of membrane proteins, and alter microRNA expression (Rezg *et al*. 2014).

Oxidative stress is caused by increased production of reactive oxygen species, or a decrease in antioxidant defense abilities. BPA has been shown to disturb oxidative homeostasis through mitochondrial function, modification of antioxidant enzymes, and increasing available acid reactive substances (products of degradation of unstable lipid peroxidases) (Rezg *et al*. 2014). Additionally, oxidative stress is a known activator of the cellular stress response (Kültz 2003).

Cell signaling to induce apoptosis is the final mechanism by which BPA affects the cell. BPA increases intracellular calcium levels and phosphorylation of kinases and nuclear translocation factors involved in apoptosis. Hormone interference also occurs at the signaling level; BPA activates kinases that disrupt hormonal mechanisms such as cellular response to estrogen (Rezg *et al*. 2014).

Other Bisphenols

Given the large body of work supporting the damaging effects of bisphenol A, some plastics manufacturers have adopted different bisphenols as the main component in plastics. These plastic products are marketed as "BPA free", though they instead contain only slightly variated bisphenols that are structural analogues of BPA, such as BPS or BPF. Both BPS and BPF have been increasingly found in a variety of personal care products, paper products, and food, and have been detected in samples from several different environmental sources (Rochester

& Bolden, 2015). Furthermore, these compounds have been detected in urine samples at levels comparable to BPA (Rochester & Bolden, 2015). One study that analyzed the presence of BPS in urine samples from 8 countries including the U.S. found that 81% of the samples contained BPS (Liao et al. 2012). These findings indicate that the substitution of BPA for BPF and BPS has led to increased concentrations and frequencies of the substitute compounds in both the environment and in the general population.

Despite their increasing use as BPA alternatives use due to health concerns, neither BPS nor BPF have been shown to be safer alternatives to BPA (Eladek *et al*. 2015). There is currently little information available about the effects of BPA substitutes on health; however, since these compounds are structural analogues, they also have the potential to have similar effects as BPA on biological systems (Rosenmai et al. 2014). For instance, studies that have examined the hormonal activities of BPF and BPS have found that they may have endocrine-disrupting effects that are similar to BPA (Rochester & Bolden 2015; Eladak *et al*. 2015).

Research on the effects of BPA, BPF, and BPS on stress granule formation previously have used a GFP-tagged G3BP protein as a marker, because of its presence in nearly all SG's. Research has shown that both BPA and BPF cause significant stress granule formation at acute high dose exposure (Salerno *et al*. 2016). BPS was not found to cause significant stress granule formation, even at high doses (Salerno *et al*. 2016). The specific mechanisms for these responses and the phosphorylation state of eIF2α in response to BPF and BPS are unclear, and it is unknown what specific stressors these compounds mimic.

Our project aimed to determine eIF2α phosphorylation state in response to treatment with BPA, BPF, and BPS, and to determine the specific eIF2α kinase(s) responsible for stress granule induction in response to treatment with each bisphenol. We hypothesized that stress granule formation in response to bisphenols (BPA, BPF, and BPS) is the result of a phosphorylation of the eIF2α translation initiation factor, and that at least one of the four eIF2α kinases is involved in this pathway. Additionally, as bisphenols have been shown to have estrogen-like effects, we predicted that cell lines containing estrogen receptors may respond differently to treatment with the bisphenols than cell lines that do not contain estrogen receptors.

Materials and Methods

Cell Line Maintenance

Double-stable osteosarcoma (U2OS-DS) cells containing GFP-G3BP and RFP-DCP1 (Kedersha *et al.* 2008), T47D breast cancer cells, and various chronic myelogenous leukemia-derived HAP1 cell lines (Aulas *et al.* 2017) were maintained. Complete DMEM media (DMEM with 10% FBS, 1% Penicillin/Streptomycin and 1% glutamine) was used for the U2OS-DS and HAP1 lines, while complete DMEM with 1% insulin was used for the T47D cells. Cells were incubated at 37°C with 5% CO_2 and sub-cultured approximately every other day at a ratio of 1:4 or 1:6 (U2OS-DS), 1:3 or 1:4 (T47D), or 1:10 (HAP1).

Acute Exposure Assays

U2OS-DS, T47D, or HAP1 cells were plated in a 12-well plate with coverslips at 8×10^4 - 1.2×10^5 cells/well. Each well contained 1 mL media. The plate was incubated for ~48 hours at 37˚C. See Figure 1 below for the plate arrangements used. 0.5 mL media was taken from each of the wells and combined with media from other wells that received the same treatment or contained the same cell line. Media was either mixed with methanol, sodium arsenite, BPA, BPF, or BPS to a final concentration of 500 μM, or left untreated. The remaining media was aspirated from each well, 0.45 mL of the treated media was returned to the appropriate wells, and the plate was incubated at 37˚C for 1 hour.
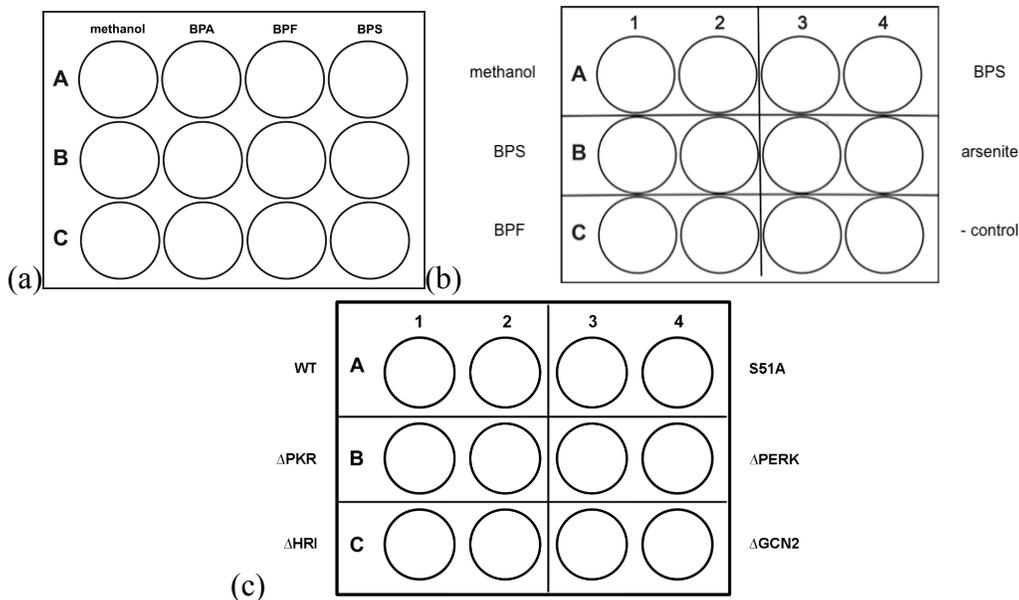
Figure 1: Acute exposure assay plate arrangement used for (a) U2OS-DS cells, (b) U2OS-DS and T47D cells, and (C) HAP1 cell lines

Media was aspirated after incubation, and the wells were rinsed with non-sterile 1X PBS. About 0.5 mL 4% paraformaldehyde was added to each well, and the plate was incubated at room temperature on an orbital shaker for 10 minutes. The wells were then incubated with cold 100% methanol for 10 minutes, followed by two rinses with 1X PBS. Following the rinses, U2OS-DS cells were directly mounted, while T47D and HAP1 cells were first stained (see staining procedure below). Following any necessary staining, the coverslips were mounted onto glass slides (one coverslip per slide) with vinyl mounting media (Fukui *et al*. 1987). The percentage of cells positive for stress granules was calculated based on manual counts using fluorescence microscopy. The cells were viewed at 400X - 630X magnification, and a minimum of 250 cells over 4 fields were counted on each slide. To reduce bias, the labels on the slides were blinded, and the results from three independent counts were averaged.

Staining Cells for Fluorescence Microscopy
Following the acute exposure assay and fixing of the cells with paraformaldehyde, each well was treated with 0.5 mL of a 5% NHS (normal horse serum) in PBS blocking solution, and the plate was incubated on a rotator for 1 hour. The blocking solution was removed from the wells, 0.5mL of the primary antibody solution (Table 1) was added to each well, and the plate was incubated on a rotator for 1 hour. The antibody was then removed and saved, and the wells were washed three times by addition of 1X PBS and incubation on a rotator for five minutes. The PBS was removed between washes and after the final wash. 0.5mL of the secondary antibody was then added to each well (Table 1). The secondary stain was applied for 1 hour while the plate was incubated on an orbital shaker. The secondary stain was then removed, and wells were washed three times with 1X PBS as before. The coverslips were then mounted as described above.

Western Blot from acute exposure assay
An acute exposure assay was performed on U2OS-DS cells with a plate arrangement as shown in Figure 1b.

Sample Collection:
Media was aspirated after 1 hour incubation with the treatments, and the wells were rinsed twice with 1X PBS, which was then aspirated fully. Each well was treated with 100 μL of 2X SDS sample buffer containing 10 mM DDT. The samples buffer were collected from each well into microcentrifuge tubes via scraping with a rubber policeman. Labelled samples were stored at -80°F.

Gel electrophoresis and Blotting:
Frozen samples were heated at 80˚C for ~15 minutes. 10 μL of each sample was loaded into a polyacrylamide gel (4-20%, BioRad). The gel was run at 120 mV for about 45-50 minutes. The proteins were transferred to PVDF membrane via electro blotting at 80 mV for 80 minutes. The presence of transferred protein was visualized using Ponceau red stain. The membrane was

blocked in a solution of 5% dry milk and wash buffer (0.05% Tween-20 in 1X PBS) overnight. The membrane was treated with one of the two primary antibodies used (see Table 1) and stored at 4˚C overnight. The membrane was then washed with wash buffer and treated with the secondary antibody (see Table 1) for 1 hour. One 5-minute rinse and three 10-minute rinses with wash buffer were carried out before 1 mL of each developing solution (from SuperSignal West Pico Trial Kit, Prod #34079) was added onto the membrane and allowed to develop for at least 2 minutes. The blot was then imaged on a Bio Rad ChemiDoc XRS+ System and analyzed using Bio Rad Image Lab software.

Table 1: Antibodies and Dilutions Used in this Study

Antibody	Application	Dilution	Manufacturer	Product Number
mouse-anti-G3BP	Immunofluorescence primary stain	2 μL/mL in 5% NHS/PBS	Santa Cruz Biotechnology	I0617
anti-mouse IgG Alexa Fluor 488 (green) stain	Immunofluorescence secondary stain	1 μL/mL in 5% NHS/PBS	Cell Signaling Technology	4408S
Hoechst 33342 (blue) nuclear stain	Immunofluorescence secondary stain	1 μL/mL in 5% NHS/PBS	Life Technologies	1642791
rabbit-anti-p-eIF2α	Western Blot primary stain	2 μL/mL in 5% dry milk in wash buffer	Cell Signaling Technology	3398T
rabbit-anti-eIF2α	Western Blot primary stain	2 μL/mL in 5% dry milk in wash buffer	Cell Signaling Technology	5324T
HRP-linked anti-rabbit IgG	Western Blot secondary stain	2 μL/mL in wash buffer	Cell Signaling Technology	7074P2

Results

BPA, BPF and BPS acute exposure comparison assay

   An acute exposure assay was performed on U2OS-DS cells to determine the percentage of cells expressing stress granules in response to treatment with BPA, BPS, or BPF, with methanol treatment used as a negative control. As shown in Figure 2, when viewed under the fluorescence microscope, cells expressing stress granules appeared to contain many small, bright dots within their cytoplasm, while the cytoplasm of cells that did not contain stress granules appeared to be a more diffuse, consistent green. This visual difference was used to count and determine the percentage of cells that had formed stress granules in response to each of the treatments.
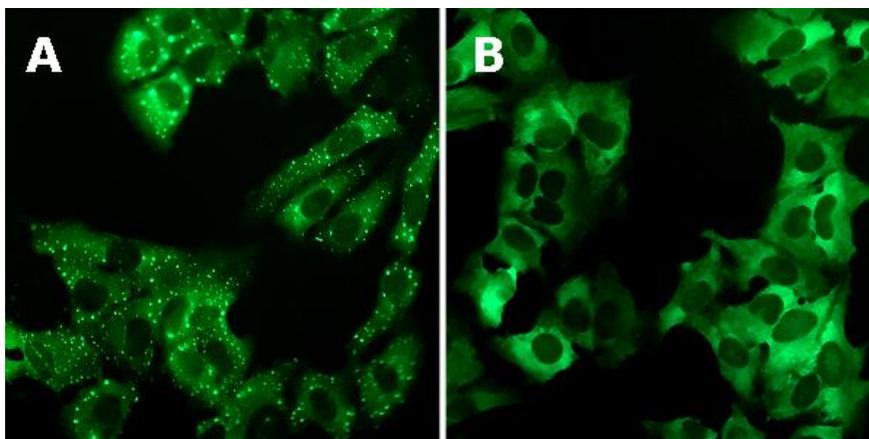
Figure 2: U2OS-DS cells that (A) contain stress granules (B) do not contain stress granules

   The results of the acute exposure assay can be seen in Figure 3 below. It was found that 99.3% of cells treated with BPA formed stress granules (the largest percentage of the three bisphenols). Cells responded more variably to BPF, in response to which 10.6% of the cells formed stress granules. Only 1.2% of cells treated with BPS formed stress granules, which was comparable to the negative control of 1.4%. These results were highly consistent with previously obtained data (Salerno *et al*. 2016; Henry *et al*. 2015), indicating that the cells and reagents were performing in our hands as expected. BPA caused the highest levels of stress granule formation, followed by BPF. BPS resulted in the lowest percentage of cells expressing stress granules of the three bisphenols.
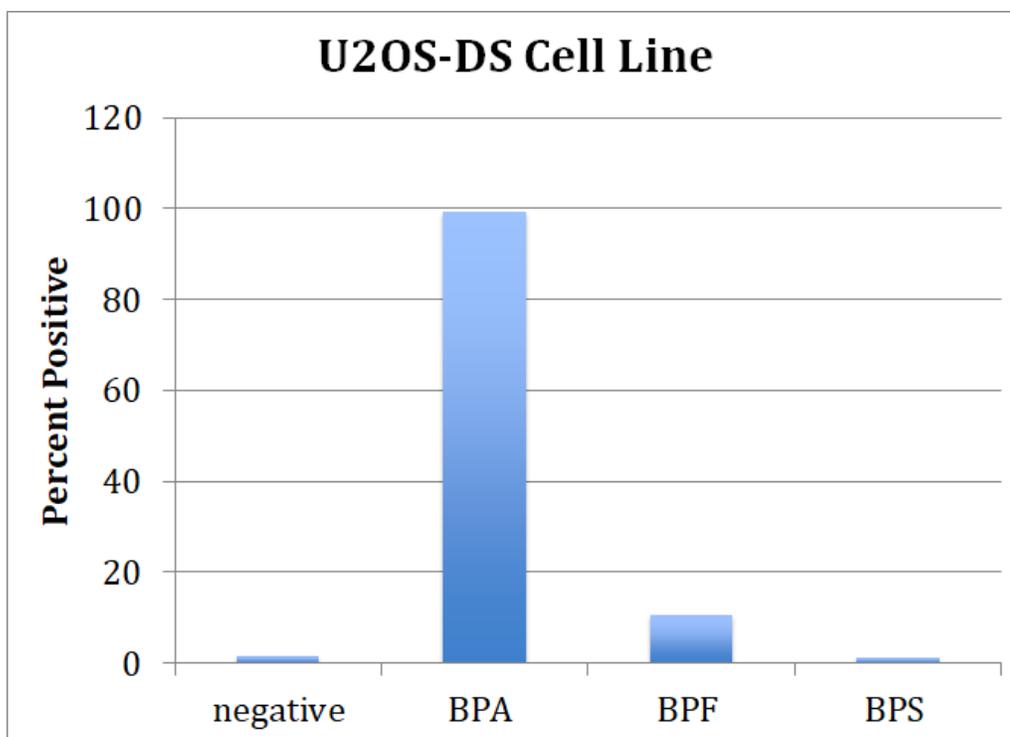
Figure 3: Stress granule formation in response to acute exposure of U2OS-DS cells to various bisphenols. Cells were treated with 500 μM solutions of the indicated bisphenols for 1 hour. (n=1)

BPA, BPF BPS, arsenite, and methanol acute exposure comparison assay

As bisphenols such as BPA are known to have estrogenic effects and are capable of binding to estrogen receptors, it was hypothesized that cells containing the estrogen receptor may respond differently to treatment with bisphenols than those that do not express the ER, such as the U2OS-DS line. In order to test this, an acute exposure assay was performed on T47D cells, which express ERβ, to determine the percentage of cells expressing stress granules in response to treatment with BPA, BPS, or BPF. Methanol treatment and no added treatment were used as negative controls, and arsenite treatment was used as a positive control. As for the U2OS-DS cells, stress granules could be visualized as dots within the cytoplasm, allowing the number of cells expressing stress granules following each treatment to be counted and the percentages to be determined. Figure 4 shows a representation of the appearance of the cells under the fluorescent microscope following treatment and staining.
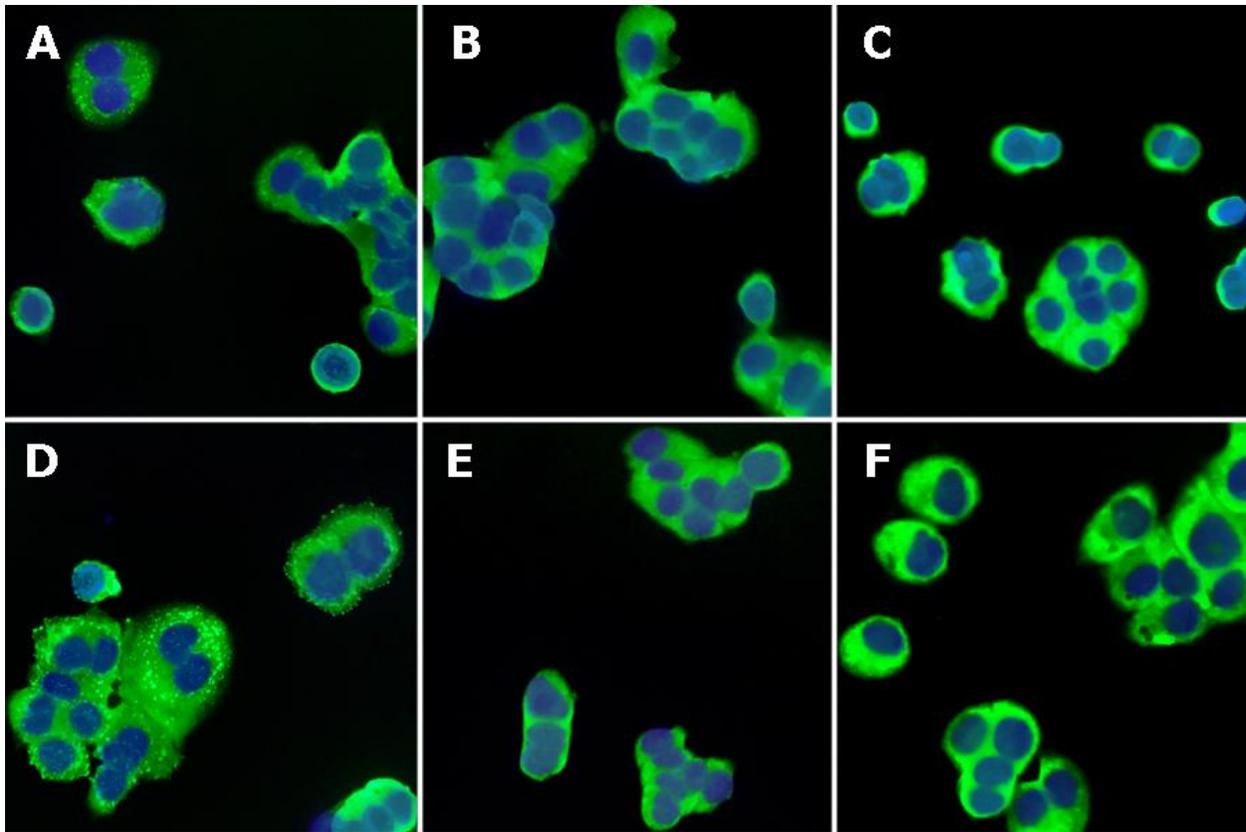
Figure 4: Composite images of T47D cells from green and blue channels on the fluorescence microscope, following a one hour treatment with (A) BPA, (B) BPF, (C) BPS, (D) arsenite, (E) methanol, and (F) negative control. Nuclei appear blue, while stress granule proteins (G3BP) are green.

The results of the acute exposure assay can be seen in Figure 5 below. Both negative controls showed low percentages of stress granule formation (0.4% and 0.9%), while 98.9% of cells treated with arsenite formed stress granules. Among the bisphenols, it was again found that the BPA treatment showed the largest percentage of cells to form stress granules at 51.6%, while BPF and BPS followed at 31.4% and 0.6%, respectively. While 99.3% of the U2OS-DS cells formed stress granules after treatment with BPA, on average only 51.6% of the T47D cells formed stress granules following the same treatment. Although four separate trials of this assay were performed, the percentage of stress granules formed in each trial was highly variable in the BPA and BPF treatment conditions, as indicated by the large error bars. While the three bisphenols again produced a similar trend in the percentages of cells that they induced to form stress granules, where BPA caused the highest stress granule formation followed by BPF and then BPS, the percentages differed from those seen in the U2OS-DS cells, particularly as a much lower percentage of stress granule formation was seen after BPA treatment of the T47D cells.
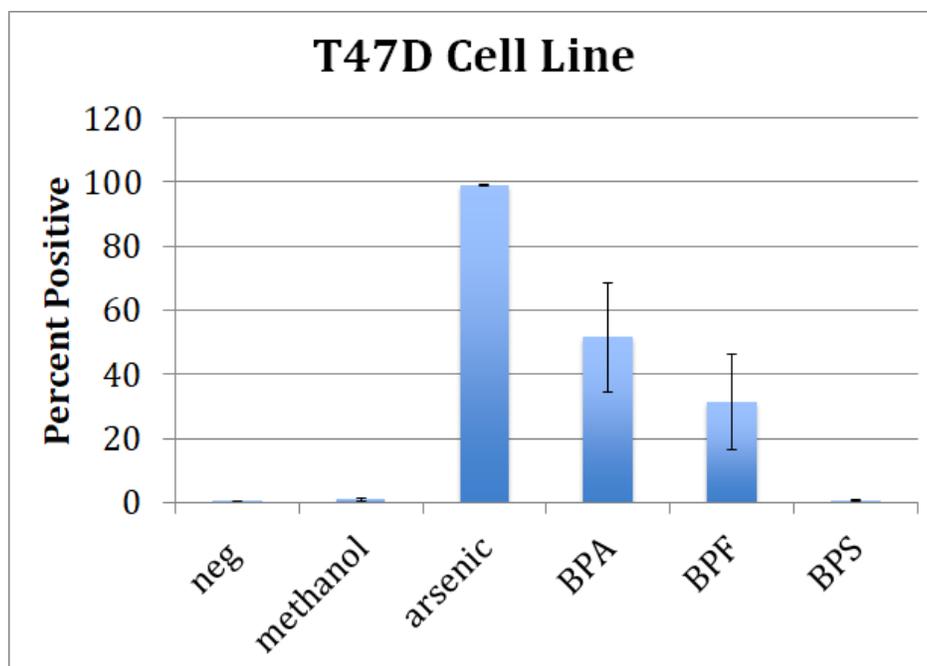
Figure 5: Stress granule formation in response to acute exposure of T47D cells to bisphenols. Cells were treated with 500 μM solutions of the indicated bisphenols or arsenite for 1 hour. Error bars represent standard error. (n=4)

Western Blot from acute exposure assay

To determine the phosphorylation state of eIF2α in response to each of the treatments, U2OS-DS cells were treated with bisphenols, and a series of Western blots were performed on the cell extracts. The results of the stain for total and phosphorylated eIF2α can be seen below in Figure 6. The black bands in the bottom row represent total eIF2α, and appear to have a consistent width across all lanes, indicating an equal protein load. The top row of Figure 6 shows the phosphorylated form of eIF2α. In this row, lanes 1-4 and 11-12 (the negative control and BPS lanes) show only faint bands, while lanes 5-8 (arsenite and BPA treatments) show dark bands. Lanes 9 and 10, corresponding to the BPF treatments, show bands of intermediate intensity. This indicates that BPA and arsenite cause high levels of eIF2α phosphorylation, BPF causes intermediate levels of eIF2α phosphorylation, and BPS causes levels of eIF2α phosphorylation that are only slightly elevated from the absence of phosphorylation seen in the negative controls.
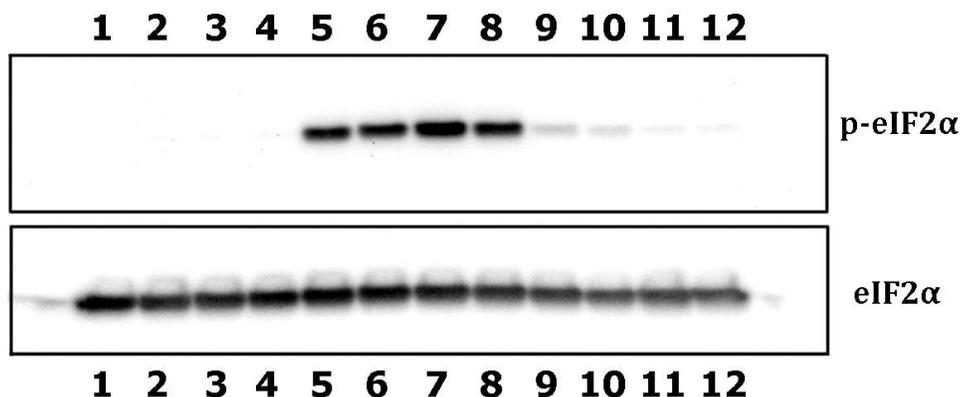
Figure 6: Western blot of acute exposure assay U2OS-DS cell extracts, stained for phosphorylated eIF2α (top) and total eIF2α (bottom). Cells treated with: negative control (lanes 1 and 2), methanol (lanes 3 and 4), arsenite (lanes 5 and 6), BPA (lanes 7 and 8), BPF (lanes 9 and 10), and BPS (lanes 11 and 12). The blots shown were selected as blots produced from three biological replicates.

BPA acute exposure comparison assay for HAP1 cells

To determine which kinase was responsible for the eIF2α phosphorylation observed in response to BPA in the Western blot results, an acute exposure assay was performed on 6 HAP1 cell lines. One line was WT cells (serving as a negative control), the second S51A line contains a mutation of the eIF2α phosphorylation site that prevents phosphorylation and thus stress granule formation (serving as a positive control), and the four remaining cell lines each contain a CRISPR knockout of one of the four kinases potentially responsible for eIF2α phosphorylation (Aulas *et al.* 2017). The cell line containing the eIF2α kinase that most strongly responds to BPA stress would be expected to exhibit a lower percentage of cells expressing stress granules in response to treatment with BPA.

As for the U2OS-DS cells, stress granules could be visualized as dots within the cytoplasm, allowing the number of cells expressing stress granules following each treatment to be counted and the percentages to be determined. Figure 8 shows a representation of the appearance of the cells under the fluorescent microscope following treatment and staining.
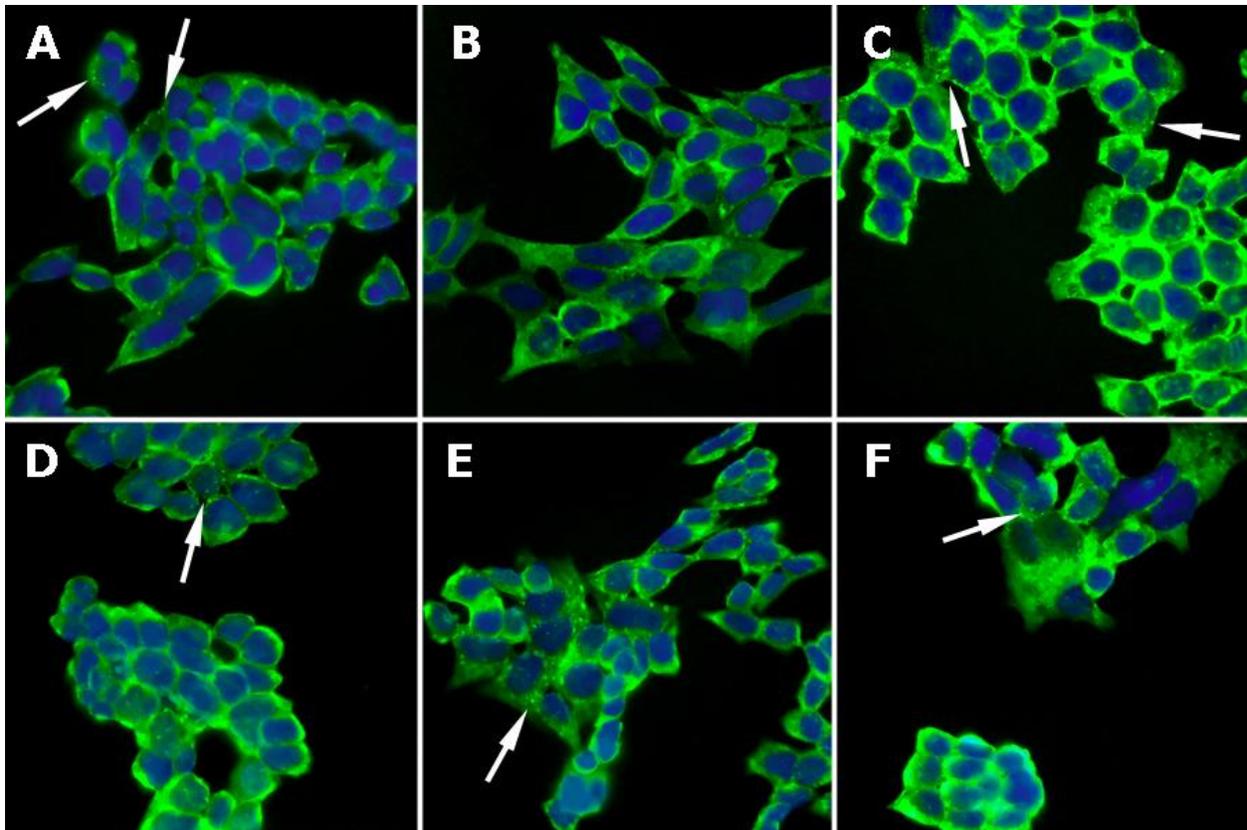
Figure 8: Composite images of HAP1 cells from green and blue channels on the fluorescence microscope, following a one-hour treatment with 500 μM BPA. Cell lines shown are (A) Wild type, (B) S51A, (C) ΔPKR, (D) ΔHRI, (E) ΔPERK, (F) ΔGCN2. Nuclei appear blue, while stress granule proteins (G3BP) are green.

The results of the acute exposure assay can be seen in Figure 9 below. The wild type HAP1 cells, the intended negative control, showed an average rate of stress granule formation of 39.9%. The S51A cells were used as the positive control and showed a low average percentage of stress granule formation, 3.6%. The four remaining cell lines of different kinase knockouts had highly variable percentages of cells form stress granules - ΔPERK cells averaged the lowest of the four with 26.9%, ΔHRI cells averaged 47.8%, ΔGCN2 cells averaged 50.0%, and the ΔPKR cells averaged 57.1%. Additionally, although three separate trials of this assay were performed, the percentage of stress granules formed in each trial was highly variable, even with WT and S51A cells.
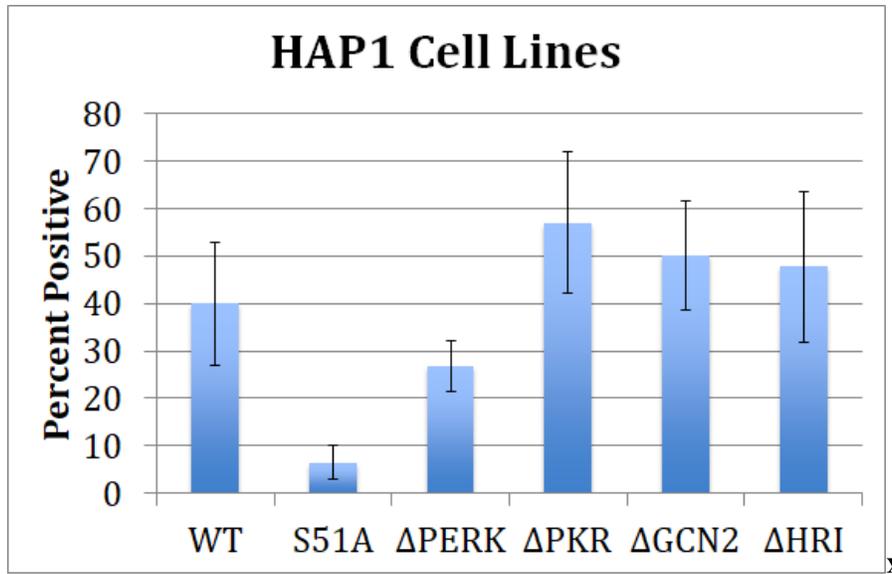
Figure 9: Stress granule formation in response to acute exposure of HAP1 cell lines to BPA. Cells were treated with 500 μM solutions of BPA for 1 hour. Error bars represent standard error. (n=3)

Discussion

U2OS-DS Acute Exposure Assay and Western Blots

When observing U2OS-DS cells overall, BPA and BPF both produced stress granules under acute exposure, while BPS did not. Of the two bisphenols, BPA treatments resulted in significantly more stress, producing the highest level of stress granule formation observed. These findings corroborate an earlier study that demonstrated that both BPA and BPF, but not BPS, cause significant stress granule formation (Salerno *et al*. 2016).

Analysis of the mechanism of stress granule formation in U2OS-DS cells was completed using Western blots performed on samples taken following acute exposure assays. Band intensities in a blot stained for phosphorylated eIF2α allow determination of the intensity of the phosphorylation state and stress granule formation response. The high rate of formation of stress granules observed in BPA- and BPF-treated U2OS-DS cells correlates to the blot bands indicating high proportions of phosphorylated eIF2α. The mechanism of stress granule formation in response to BPA and BPF stress appears to be eIF2α phosphorylation, which prevents translation initiation. Relative intensity of the bands for each treatment correlated with observed rates of stress granule formation.

Despite the low levels of SG formation observed in BPS, and the variability seen in the BPF trials, the risks that these bisphenols possess cannot be ruled out. The experiments used uniform concentrations of the bisphenol solutions, which may differ from the actual exposure level of bisphenols in daily life. Our findings suggest that even the alternative bisphenols may be harmful to health as, like BPA, they also appear capable of triggering the cellular stress response via the same pathway. Further investigation into the pathways triggered by the bisphenols may continue to elucidate the specific health impacts of these compounds, while the development of non-bisphenol BPA alternatives may reduce the risks of widespread bisphenol usage.

HAP1 Acute Exposure Assay

Following identification of the mechanism of stress granule formation as eIF2α phosphorylation, the following assay aimed to identify the kinase responsible for the phosphorylation event. PKR-like ER kinase (PERK) knockout cell lines exhibited the lowest rates of stress granule formation of the four knockouts (26.9%), and was the only knockout cell line that had a significant reduction in SG formation compared to the WT cells. This suggests that PERK may be playing the largest role in the phosphorylation of eIF2α that causes stress granules. PERK has been associated with responses to endoplasmic reticulum stress, though BPA is known to operate through an oxidative stress pathway (Rezg *et al*. 2014).

Compared to the U2OS-DS cell line, highly variable rates of stress granule formation were observed in each of the six HAP1 cell line, both in kinase knockouts and controls (WT and S51A). In the four HAP1 knockouts the stress granule formation rates were between 26.9% (ΔPERK) and 57.1% (ΔPKR). This suggests that a combination of kinases were acting to phosphorylate eIF2α in cells under BPA treatment. This could be indicative of the complex

mechanism of cellular stress response to BPA in general, or be an aspect of HAP1 cells alone. Different cell types do not have identical response to BPA; for instance, the T47D cells showed overall lower stress granule formation rates in comparison to U2OS-DS cells at identical concentrations. WT HAP1 cells, which were expected to serve as the positive control, did not exhibit consistent or high rates of stress granule formation. A BPA, arsenite, and methanol chronic exposure assay of WT HAP1 cells could determine baseline rates of stress granule formation in treated cells.

When the HAP1 cell lines were examined, the ΔPERK trials were the only ones showing considerable reduction in SG formation compared to WT. This indicates that PERK (usually active in endoplasmic reticulum stress) was important in SG formation triggered by the bisphenols treatment, while it remains unclear if the other kinases may also play a role in this process. The finding that the kinase knockout cell lines such as ΔPKR, ΔGCN2, and ΔHRI appeared to produce more SG's than the WT line is also worth further investigation. This may indicate that the HAP1 cells have an inherent difference in their ability to form SGs, or the phenomenon may be due to chance. An analysis of significance may elucidate this. Future studies could focus on the WT HAP1 cells, and establish a reliable baseline for comparison with the kinase knockouts.

T47D Acute Exposure Assay

Since bisphenols have been shown to have estrogenic effects, this study also investigated whether a cell line that contains estrogen receptors would respond differently to the bisphenols in terms of stress granule formation than the U2OS-DS cell line, which does not have estrogen receptors. The T47D cell line, which has the estrogen receptor α (ERα), was used for this purpose. The comparison of the acute exposure assay results for the two cell lines identifies some slight differences in the percentage of cells expressing stress granules following treatment with the bisphenols. Unlike in the U2OS-DS cells, where BPA caused nearly 100% of cells to form stress granules, only around 52% of the T47D cells showed stress granules after BPA treatment. Additionally, while only around 11% of BPF-treated U2OS-DS cells formed stress granules, this number was increased to 31% in T47D cells. A comparably low percentage of cells treated with BPS formed stress granules in both cell lines. Due to the variability of the cell counts between trials, particularly with respect to BPA and BPF, four trials instead of the typical three were completed. This variability could be a result of the presence of hormone receptors in T47D cells - it is likely a result of the endocrine disrupting effects of bisphenols because this variability was not observed in the positive and negative controls. Hormone receptors affect cell growth and metabolism, and even slightly varied metabolic rates could yield vastly different responses to stress in cells. More exploration may be necessary to clarify the variability of the effects of the two bisphenols in this cell line.

The T47D trials gave slightly different results compared to the U2OS-DS trials. The apparent lower number of SGs in BPA trials and higher number in BPF trials may suggest different sensitivity levels to the two bisphenols, which may be related to the presence of the

estrogen receptor in this cell line. However, due to the greater variability seen in the BPF trials, it is ambiguous whether the T47D cell are indeed more sensitive to BPF. There is also a possibility that the difference in T47D and U2OS-DS trials is not related to the estrogen receptor, but to other characteristics of the cell line. This should be explored further using other estrogen receptor-positive cell lines.

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Appendix A: Raw Data for U2OS-DS acute exposure assay

cell type	# stressed	# not stressed	% stressed	average % stressed
negative	5	281	1.75	1.41
	4.0	301.0	1.31	
	1.0	250.0	0.40	
	0.0	206.0	0.00	
	3.0	253.0	1.17	
	6.0	256.0	2.29	
	5.0	241.0	2.03	
	3.0	281.0	1.06	
	7.0	251.0	2.71	
BPA	300.0	4.0	98.68	99.22
	266.0	0.0	100.00	
	307.0	0.0	100.00	
	267.0	6.0	97.80	
	300	3	99.01	
	244	1	99.59	
	227	3	98.70	
	236	1	99.58	
	244	1	99.59	
BPF	99	240	29.20	10.59
	12	240	4.76	
	24	278	7.95	
	74	197	27.31	
	7	239	2.85	
	7	263	2.59	
	54	250	17.76	
	2	279	0.71	
	5	223	2.19	
BPS	3	290	1.02	1.18
	3	310	0.96	
	2	249	0.80	
	1	289	0.34	
	6	244	2.40	
	1	228	0.44	
	5	219	2.23	
	3	222	1.33	
	3	261	1.14	

Appendix B: Raw Data for HAP1 cell lines acute exposure assay

cell type	# stressed	# not stressed	% stressed	average % stressed
GCN2	93	202	31.53	50.05
	81	178	31.27	
	17	244	6.51	
	137	134	50.55	
	10	245	3.92	
	103	151	40.55	
	199	62	76.25	
	225	70	76.27	
	169	87	66.02	
	208	57	78.49	
	131	269	32.75	
	245	204	54.57	
	133	141	48.54	
	112	190	37.09	
	165	76	68.46	
	199	79	71.58	
	278	99	73.74	
	185	165	52.86	
HRI	137	161	45.97	47.74
	123	127	49.20	
	147	219	40.16	
	55	305	15.28	
	17	256	6.23	
	100	190	34.48	
	21	241	8.02	
	172	83	67.45	
	10	290	3.33	
	195	89	68.66	
	89	360	19.82	
	77	265	22.51	
	229	77	74.84	
	207	82	71.63	
	237	28	89.43	
	286	43	86.93	
	291	64	81.97	
	297	108	73.33	

cell type	# stressed	# not stressed	% stressed	average % stressed
perk	44	283	13.46	26.92
	231	203	53.23	
	75	249	23.15	
	135	187	41.93	
	52	199	20.72	
	149	96	60.82	
	45	221	16.92	
	44	219	16.73	
	47	235	16.67	
	40	211	15.94	
	55	326	14.44	
	79	287	21.58	
	37	239	13.41	
	44	245	15.22	
	110	201	35.37	
	150	154	49.34	
	99	346	22.25	
	85	170	33.33	
PKR	251	122	67.29	56.73
	198	59	77.04	
	351	30	92.13	
	289	40	87.84	
	144	120	54.55	
	198	65	75.29	
	40	250	13.79	
	94	178	34.56	
	66	224	22.76	
	152	136	52.78	
	44	348	11.22	
	102	250	28.98	
	183	88	67.53	
	190	87	68.59	
	193	80	70.70	
	233	83	73.73	
	216	113	65.65	
	201	116	63.41	

cell type	# stressed	# not stressed	% stressed	average % stressed
WT	82	173	32.16	37.27
	193	73	72.56	
	84	217	27.91	
	217	99	68.67	
	78	183	29.89	
	141	133	51.46	
	97	173	35.93	
	0	297	0.00	
	101	196	34.01	
	0	295	0.00	
	74	338	17.96	
	0	297	0.00	
	106	160	39.85	
	135	148	47.70	
	169	1000	14.46	
	215	93	69.81	
	174	137	55.95	
	185	70	72.55	
S51A	83	252	24.78	6.46
	43	227	15.93	
	26	469	5.25	
	32	321	9.07	
	17	243	6.54	
	46	216	17.56	
	0	260	0.00	
	4	252	1.56	
	3	257	1.15	
	5	259	1.89	
	0	283	0.00	
	1	332	0.30	
	5	289	1.70	
	13	276	4.50	
	8	277	2.81	
	18	260	6.47	
	29	284	9.27	
	29	357	7.51	

Appendix C: Raw Data for T47D cell line acute exposure assay

treatment	# stressed	# not stressed	% stressed	average % stressed
BPF	90	125	41.86	31.44
	127	93	57.73	
	21	256	7.58	
	120	161	42.70	
	79	262	23.17	
	92	245	27.30	
	5	227	2.16	
	2	233	0.85	
	3	258	1.15	
	7	238	2.86	
	2	311	0.64	
	3	260	1.14	
	164	55	74.89	
	164	113	59.21	
	198	14	93.40	
	172	38	81.90	
	142	59	61.80	
	123	109	58.00	
	4	237	1.66	
	101	176	36.46	
	0	249	0.00	
	105	147	41.67	
	3	311	0.68	
	97	175	35.67	

treatment	# stressed	# not stressed	% stressed	average % stressed
BPS	0	210	0.00	0.61
	0	205	0.00	
	0	286	0.00	
	0	255	0.00	
	1	222	0.45	
	1	261	0.38	
	1	218	0.46	
	0	232	0.00	
	3	251	1.18	
	6	244	2.40	
	5	269	1.82	
	4	293	1.35	
	6	247	2.37	
	0	215	0.00	
	1	233	0.43	
	0	230	0.00	
	0	245	0.00	
	1	244	0.40	
	1	243	0.41	
	3	244	1.21	
	2	255	0.78	
	1	290	0.34	
	1	263	0.36	
	1	212	0.29	

treatment	# stressed	# not stressed	% stressed	average % stressed
arsenic	229	2	99.13	98.90
	210	5	97.67	
	291	0	100.00	
	308	0	100.00	
	225	2	99.12	
	232	3	98.72	
	205	3	98.56	
	224	5	97.82	
	222	3	98.67	
	223	7	96.96	
	281	2	99.29	
	268	7	97.45	
	279	0	100.00	
	274	0	100.00	
	223	3	98.67	
	238	4	98.35	
	221	3	98.60	
	282	2	99.70	
	236	4	98.33	
	249	0	100.00	
	247	4	98.41	
	302	3	99.02	
	270	3	99.47	
	253	2	99.69	

treatment	# stressed	# not stressed	% stressed	average % stressed
BPA	161	63	71.88	51.75
	120	99	54.79	
	143	130	52.38	
	138	121	53.28	
	161	73	68.80	
	138	123	52.87	
	156	54	74.29	
	141	71	66.51	
	102	121	45.74	
	90	124	42.06	
	128	126	50.39	
	100	207	32.57	
	219	25	89.75	
	218	29	88.26	
	205	19	91.52	
	193	18	91.47	
	247	49	84.80	
	205	17	90.70	
	15	273	5.21	
	50	248	16.78	
	4	313	1.26	
	11	214	4.89	
	11	213	4.80	
	20	253	6.97	

treatment	# stressed	# not stressed	% stressed	average % stressed
negative control	1	250	0.40	0.41
	0	233	0.00	
	0	266	0.00	
	0	367	0.00	
	6	220	2.65	
	2	271	0.73	
	2	229	0.87	
	2	206	0.96	
	0	235	0.00	
	1	235	0.42	
	0	292	0.00	
	2	288	0.69	
	0	231	0.00	
	4	218	1.80	
	0	243	0.00	
	0	209	0.00	
	0	212	0.00	
	2	270	0.70	
	0	263	0.00	
	1	232	0.43	
	0	263	0.00	
	0	252	0.00	
	0	272	0.00	
	0	236	0.00	

treatment	# stressed	# not stressed	% stressed	average % stressed
methanol	0	244	0.00	0.89
	0	233	0.00	
	10	272	3.55	
	3	286	1.04	
	8	237	3.27	
	7	222	3.06	
	1	272	0.37	
	3	213	1.39	
	1	232	0.43	
	7	218	3.11	
	0	265	0.00	
	5	292	1.68	
	0	247	0.00	
	2	223	0.89	
	1	213	0.47	
	1	216	0.46	
	0	253	0.00	
	1	260	0.30	
	2	249	0.80	
	1	288	0.35	
	0	214	0.00	
	1	242	0.41	
	1	290	0.34	
	1	306	0.28	